

Induction of oxidative DNA damage by mesalamine in the presence of copper: A potential mechanism for mesalamine anticancer activity

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Abstract:

Mesalamine is the first line pharmacologic intervention for patients with ulcerative colitis, and recent epidemiologic studies have demonstrated a protective association between therapeutic use of the drug and colorectal carcinoma. However, the mechanism by which this protection is afforded has yet to be elucidated. Because copper is found at higher than normal concentrations in neoplastic cell nuclei and is known to interact with phenolic compounds to generate reactive oxygen species, we investigated whether the reaction of mesalamine/copper was able to induce oxidative DNA strand breaks in ϕ X-174 RF I plasmid DNA, and the various components of the mechanism by which the reaction occurred. Plasmid DNA strand breaks were induced by pharmacologically relevant concentrations of mesalamine in the presence of a micromolar concentration of Cu(II), and damage was inhibited by bathocuproinedisulfonic acid (BCS) and catalase. Further, we showed that the reaction of copper with mesalamine consumed molecular oxygen, which was inhibited by BCS. Electron paramagnetic resonance spectral analysis of the reaction of copper/mesalamine indicated the presence of the hydroxyl radical, which was inhibited by both BCS and catalase. This study demonstrates for the first time that through a copper-redox cycling mechanism, the copper-mediated oxidation of mesalamine is a pro-oxidant interaction that generates hydroxyl radicals which may participate in oxidative DNA damage. These results demonstrate a potential mechanism of the anticancer effects of mesalamine in patients with ulcerative colitis.

BCS, bathocuproinedisulfonic acid;

5-ASA, 5-acteylsalicylic acid, mesalamine;

POBN, α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone;

PBS, phosphate buffered saline;

EPR, electron paramagnetic resonance;

ROS, reactive oxygen species

Keywords: Mesalamine | Copper | DNA strand breaks | Anticancer activity

Article:

1. Introduction

It has been well established that chronic inflammation is a cause of dysregulated cell growth and may both initiate and propagate the steps of carcinogenesis (Fitzpatrick, 2001 and Shacter and Weitzman, 2002). Patients with ulcerative colitis experience such chronic inflammation of the bowel and are known to be at an increased risk for colorectal carcinoma (Kulaylat and Dayton, 2010), which remains the third leading cause of cancer death among both men and women in the United States (Jemal et al., 2010). Mesalamine (5-aminosalicylic acid, 5-ASA, Fig. 1) has proven to be an effective first-line non-steroidal anti-inflammatory pharmacologic therapy of mild-moderate ulcerative colitis in man (Karagozian and Burakoff, 2007), and several recent epidemiologic studies have shown a protective association between the use of 5-ASA in these patients and the development of colorectal carcinoma (Lyakhovich and Gasche, 2010 and Velayos et al., 2005). However, the mechanism by this anticancer effect is afforded remains yet to be determined.

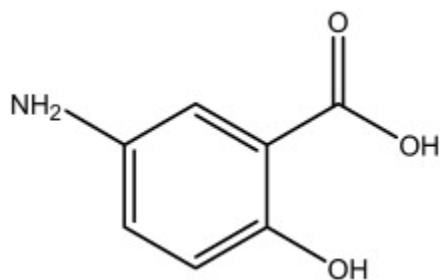


Fig. 1. Structure of mesalamine.

Several studies point to the interaction of phenolic compounds, transition metals, and induction of reactive oxygen species (ROS) as the potential origin of such anticancer activity (Hadi et al., 2007, Li et al., 2002, Li and Trush, 1994, Wang et al., 2008 and Win et al., 2002). A number of phenolic compounds have demonstrated anticancer effects by ROS scavenging or antioxidant

activity inhibiting generation of the potent radicals (Cao and Li, 2004, Hsu and Li, 2002 and Li and Cao, 2002). However, recent studies have shown that some phenolic compounds in fact exhibit pro-oxidant activity, leading to oxidative DNA damage, particularly in the presence of copper (Wang et al., 2008).

It has been well established that concentrations of cellular copper are elevated in various malignancies (Hadi et al., 2007), and that the redox-active transition metal exists in the nucleus, closely associated with DNA bases, particularly at G-C sites (Geierstanger et al., 1991 and Li and Trush, 1994). Studies have further shown that copper may bioactivate phenolic compounds to generate ROS (Hadi et al., 2007, Li and Trush, 1994 and Oikawa and Kawanishi, 1998) and thereby may mediate oxidative DNA damage that results in apoptotic DNA fragmentation or other cell death modes (Cochrane, 1991, Hadi et al., 2007, Li and Trush, 1994, Oikawa and Kawanishi, 1998 and Wang et al., 2008). Specifically, Hadi et al. (2007) demonstrated that in the phenolic compounds quercetin, curcumin, and others exhibit pro-oxidant activity in the presence of copper and Li and Trush (1994) demonstrated pro-oxidant activity of phenolic compounds catechol, caffeic acid, eugenol, and others when in the presence of copper.

Recently, Koelink et al. (2009) studied the effect of treating isolated colorectal cancer cell lines with the phenolic compound mesalamine, and observed dose-dependent cell-cycle arrest which induced apoptosis and mitotic catastrophe. Given these findings, we hypothesized that 5-ASA would react with copper and manifest pro-oxidant activity, potentiate reactive oxygen species formation, and induce oxidative damage in a plasmid DNA system. Based on our observations of the various steps in the reaction of Cu(II)/5-ASA, we propose for the first time a mechanism by which mesalamine may potentiate oxidative DNA damage and may be responsible for its epidemiologically demonstrated anticarcinogenic activity.

2. Materials and methods

2.1. Materials

φX-174 RF I plasmid DNA was from New England Biolabs, Beverly, MA. Phosphate buffered saline (PBS) was from Gibco Life Technologies, Grand Island, NY. Alpha-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) and DMSO were from Aldrich Chemical Co. (Milwaukee, WI). Mesalamine, bathocuproinedisulfonic acid (BCS), cupric sulfate, catalase, and all other chemical reagents were from Sigma Chemicals, St. Louis, MO.

2.2. Assay for oxidative DNA strand breaks

DNA strand breaks were measured by the conversion of φX-174 RF I double stranded DNA to open circular and linear conformations, according to the procedure described previously (Li and Trush, 1993). In brief, 6 μl plasmid DNA was incubated at 37 °C for 30 min with increasing concentrations of mesalamine in the presence or absence of copper in PBS (pH 7.4) at a final volume of 24 μl. Further, 6 μl plasmid DNA was incubated with 5-ASA and Cu(II) in the

presence or absence of BCS or increasing concentrations of active or heat-inactivated catalase in PBS (pH 7.4) at 37 °C for 30 at a final volume of 24 μ l. Following incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris base, 20 mM sodium acetate, and 2 mM EDTA, and electrophoresed in a horizontal slab gel apparatus with Tris/acetate/EDTA gel buffer at 150 V for 1 h. After electrophoresis the gels were stained with 0.5 μ g/ml solution of ethidium bromide for 30 min and destained in water for 30 min. The gels were then photographed under ultraviolet illumination and quantified using Alpha Innotech Imaging System (San Leandro, CA).

2.3. Measurement of oxygen consumption

Oxygen consumed by the reaction of 5-ASA/Cu(II) was monitored with a Clark oxygen electrode (YSI 500, Yellow Springs, OH) as described previously (Li et al., 1999). Briefly, experimental reagents were injected at 5 min increments into 2.5 ml air saturated PBS (pH 7.4) in a Perspex incubation chamber at 37 °C and allowed to incubate for 30 min upon the addition of the final reagent. The reagents were mixed according to the following three experimental conditions: 1, Cu(II) + 5-ASA; 2, Cu(II) + 5-ASA + BCS; 3, Cu(II) + BCS + 5-ASA.

2.4. Electron paramagnetic resonance spin-trapping assay

Hydroxyl radical formation induced by the reaction of 5-ASA/Cu(II) was determined by converting hydroxyl radical (OH) to methyl radical using DMSO, and detected by using α -(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (POBN) spin trapping electron paramagnetic spectra of methyl radicals. The electron paramagnetic resonance (EPR) spectra were recorded at room temperature using a spectrometer (Bruker D-200 ER, IBM Bruker), operating at X band with a TM cavity and capillary tube. EPR spectrometer settings were: modulation frequency 100 KHz; X band microwave frequency, 9.5 GHz; microwave power 20 mW; modulation amplitude, 6.3 G; time constant 160 s; scan time, 400 s; and receiver gain 4×10^5 . To prepare samples for EPR spectral analysis, 10 μ M cupric sulfate, 50 mM POBN, and 0.5 M DMSO were mixed in test tubes in the presence or absence of 1 mM 5-ASA, 50 μ M BCS, 100 U/ml catalase, and 100 U/ml heat-inactivated catalase at a final volume of 100 μ l. Samples were incubated at 37 °C for 30 min and then transferred to a capillary tube for EPR spectral analysis at room temperature under the above listed conditions.

2.5. Statistical analyses

Single-factor ANOVA testing was used to analyze variance, and further statistical analyses for post hoc comparisons were done by two-tailed *t*-test. All differences of $P \leq 0.05$ were considered significant.

3. Results and discussion

3.1. Induction of DNA strand breaks by the Cu(II)/5-ASA

Induction of single- and double-strand breaks to the supercoiled double stranded ϕ X-174 RF I plasmid DNA manifests in open circular and linear conformation DNA forms, respectively (Li and Trush, 1993). As shown in Fig. 2, incubation of the plasmid DNA with 10 μ M Cu(II) and 0.01, 0.1. and 1 mM 5-ASA resulted in the significant formation of open circular and linear forms of DNA in a concentration-dependent fashion. It is important to note that the concentrations of 5-ASA used in the present study are pharmacologically relevant. Frieri et al. (1999) have reported that in patients receiving both 2.4 g/day oral and 4 g/day topical 5-ASA, colonic mucosal tissue concentrations of the drug were 0.3–0.6 mM. Copper is an essential trace element, which is present in many tissues at micromolar concentrations (Hadi et al., 2007 and Li et al., 1994). Previous studies have suggested that copper level in the cancerous tissue is considerably increased up to 100 μ M (Hadi et al., 2007 and Li et al., 1994). Thus, the concentration of copper used in this study (10 μ M) is physiologically relevant. Our results as shown in Fig. 2 demonstrate for the first time that the reaction of Cu(II)/5-ASA was able to elicit both single- and double-stranded DNA breaks in this plasmid DNA system at pharmacologically relevant concentrations. Recently, Koelink et al. (2009) reported that 5-ASA induced apoptosis in isolated colorectal cancer cells suggesting that the induction of cell death by 5-ASA plays a major role in its anticancer effect. However, its mechanism remains unknown. Because oxidative DNA damage can cause irreversible cellular injury and ultimately result in cancer cell death (Hadi et al., 2007), the DNA cleaving effects of 5-ASA in the presence of Cu(II) observed in the present study may explain, at least in part, the anticancer effects of 5-ASA in patients with ulcerative colitis (Lyakhovich and Gasche, 2010). It is also worth mentioning that in a mouse model, 5-ASA was found to significantly suppress the development of colitis-associated cancer (Ikeda et al., 2007). Interestingly, 5-ASA alone only reduced tumor cell proliferation without affecting normal cells indicating that its inhibitory effect is specific for cancer cells (Ikeda et al., 2007).

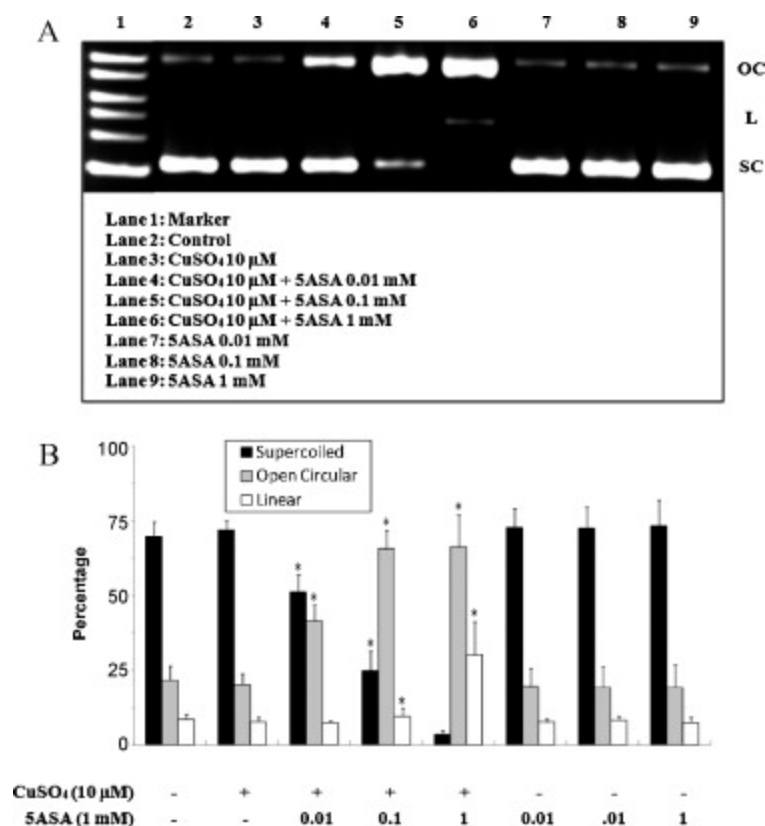


Fig. 2. Induction of DNA damage by various concentrations of mesalamine in the presence of copper. ϕ X-174 RF I plasmid DNA was incubated at 37 °C with the indicated concentrations of mesalamine in the presence or absence of copper for 30 min. (A) A representative agarose gel picture; (B) quantitative analysis of DNA damage. OC, open-circular DNA conformation; L, linear DNA conformation; SC, supercoiled DNA conformation. Data represent means \pm SD of three independent experiments. (* $P < 0.05$ vs. each of DNA conformation matched-control.)

3.2. Inhibition of Cu(II)/5-ASA-induced DNA breaks by BCS and catalase

Bathocuproinedisulfonic acid (BCS) is a known Cu(I)-specific chelating agent (Li and Trush, 1993). As can be seen from Fig. 3A and B (lane 4), the reaction of Cu(II)/5-ASA/BCS failed to induce significant open circular or linear DNA formations. This observation showed that chelation of Cu(I) ions by BCS blocked Cu(II)/5-ASA-induced DNA damage, and demonstrates that copper redox is a critical component of the reaction mechanism by which Cu(II)/5-ASA induce DNA damage.

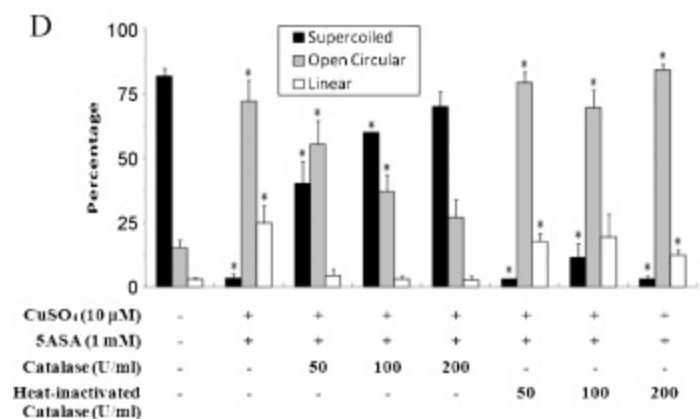
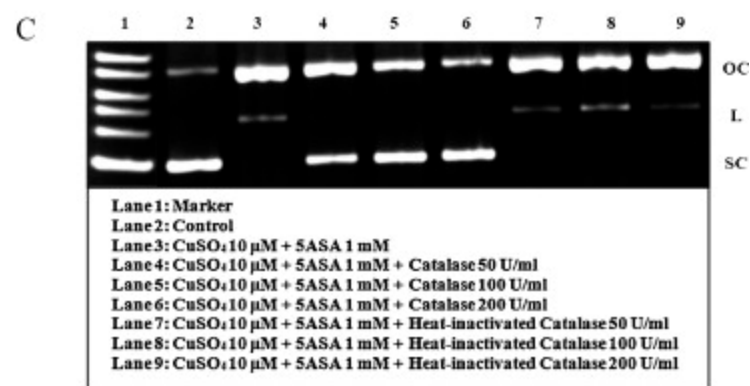
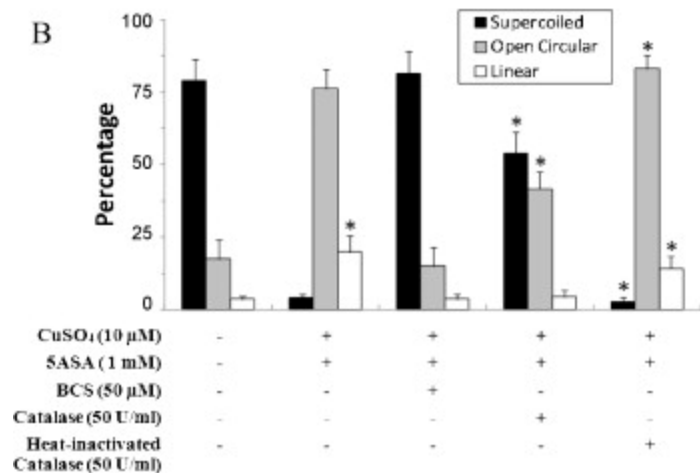
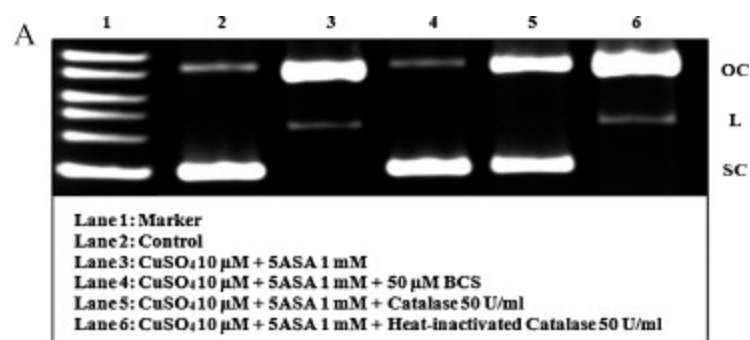


Fig. 3. Involvement of Cu(I) and H₂O₂ in mesalamine/copper-induced DNA damage. ϕ X-174 RF I plasmid DNA was incubated at 37 °C with the indicated concentrations of mesalamine and copper in the presence or absence of BCS, and the indicated increasing concentrations of catalase and heat-inactivated catalase for 30 min. (A) A representative agarose gel picture, demonstrating effects of BCS and low-concentration catalase and heat-inactivated catalase; (B) quantitative analysis of DNA damage demonstrated in panel A; (C) a representative agarose gel picture, demonstrating effects of increasing concentrations of catalase and heat-inactivated catalase; (D) quantitative analysis of DNA damage demonstrated in panel C. OC, open-circular DNA conformation; L, linear DNA conformation; SC, supercoiled DNA conformation. Data represent means \pm SD of three independent experiments. (**P* < 0.05 vs. each of DNA conformation matched-control.)

The reaction of H₂O₂ and Cu(II) is known to cause DNA strand breaks and is widely used as a model for oxidative DNA damage (Hsu and Li, 2002), while catalase is a physiologic enzyme known to catalyze the decomposition of H₂O₂ to water and oxygen and thereby inhibit this damage (Yamamoto and Kawanishi, 1989). As can be seen in Fig. 3A and B, the reaction of Cu(II)/5-ASA and 50 U/ml catalase failed to significantly induce in linear DNA formation, but did significantly increase and decrease open circular and supercoiled DNA, respectively. When heat-inactivated catalase was added to Cu(II)/5-ASA, open-circular and linear DNA formations were significantly increased. These observations showed that the action of catalase at this concentration was able to offer some incomplete protection against oxidative DNA damage. Heated, enzymatically inactive catalase failed to demonstrate any non-specific protection against DNA damage.

The protective effect of catalase was studied further at increasing concentrations. As can be seen from Fig. 3C and D, the protective effect of catalase was markedly dose-dependent: as the concentration of catalase increased, the formation of supercoiled DNA significantly increased, and open circular DNA significantly decreased; there was no significant formation of linear form DNA at any catalase concentration. The addition of various concentrations of heated, enzymatically inactive catalase demonstrated no effect on Cu(II)/5-ASA-induced oxidative DNA damage. These observations demonstrated that catalase provides a distinct dose-dependent inhibition of Cu(II)/5-ASA-induced DNA damage, and heat-inactivated catalase fails to provide non-specific protection. Further, because catalase offered protection against oxidative DNA damage, H₂O₂ formation is a critical part of the reaction mechanism responsible for the DNA damage shown above.

3.3. Cu(II)-mediated oxygen consumption in the presence of mesalamine

Because we have shown above that the copper redox and H₂O₂ formation are components of the mechanism by which the copper and mesalamine react to induce DNA damage, we investigated the consumption of O₂ by the reaction. In the presence of free radicals, molecular oxygen is known to be consumed by reduction to superoxide radicals, and consequently oxidize to form

H₂O₂ in the presence of Cu(I) ions (Yamamoto and Kawanishi, 1989). As can be seen from Fig. 4A and D, the reaction of Cu(II)/5-ASA consumed oxygen significantly. Fig. 4B shows that the addition of BCS to the incubating reaction of Cu(II)/5-ASA halts the consumption of O₂, and Fig. 4C shows that when BCS was added to the Perspex chamber before the addition of 5-ASA, the reaction of Cu(II)/5-ASA did not significantly consume O₂. These observations showed that the reaction of Cu(II)/5-ASA consumes oxygen, supporting the hypothesis that molecular oxygen is reduced to propagate the ongoing radical chemistry. The demonstrated effect of BCS further supports the argument that copper redox is a critical component of the induction of radical formation that contributes to Cu(II)/5-ASA-induced DNA damage.

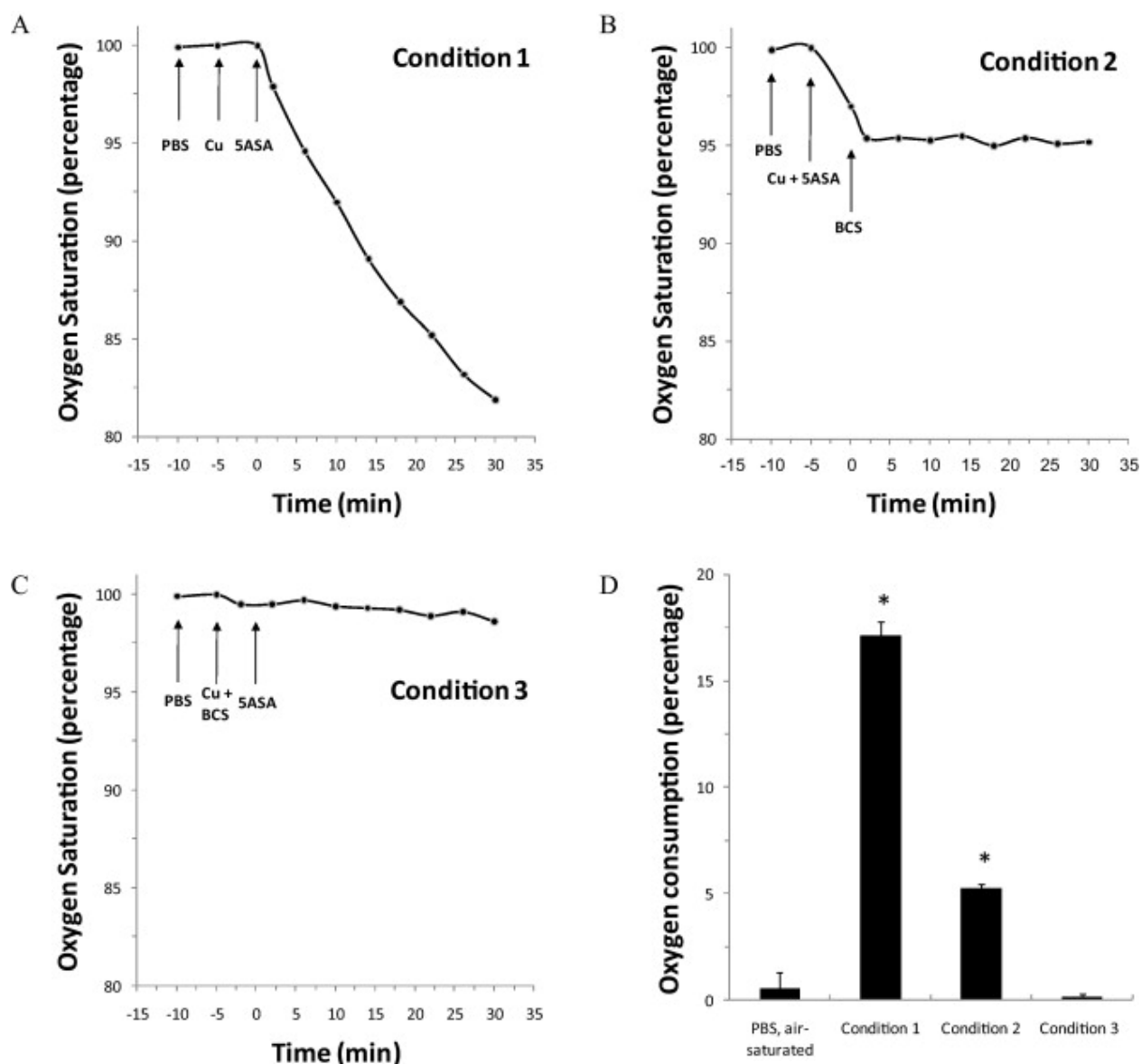


Fig. 4. Oxygen consumption induced by mesalamine/copper and role of Cu(II)/Cu(I) redox. The oxygen consumption was measured upon incubation of the 10 μ M Cu(II) and 1 mM mesalamine

in the presence or absence of 50 μ M BCS according to the following conditions. Condition 1: copper injected, incubated 5 min, 5-ASA injected, incubated 30 min. Condition 2: copper and 5-ASA injected, incubated 5 min, BCS injected, incubated 30 min. Condition 3: copper and BCS injected, incubated 5 min, 5-ASA injected, incubated 30 min. (A) The representative oxygen consumption curves for the indicated experimental conditions. (B) Effects of BCS on the oxygen consumption caused by the reaction of copper and mesalamine; data represent means \pm SD of three independent experiments. (* $P < 0.05$ vs. control.)

3.4. Generation of hydroxyl radical from the reaction of Cu(II)/5-ASA

Since both H_2O_2 and a Cu(II)/Cu(I) redox cycle were shown to occur during the Cu(II)-mediated oxidation of 5-ASA (Fig. 2, Fig. 3 and Fig. 4), the hydroxyl radical may be generated from the reaction of H_2O_2 and Cu(I). Further, it has been shown previously that OH are causally associated with the single- and double-stranded DNA breaks seen in oxidative DNA damage (Li and Trush, 1993). Accordingly, EPR spin trapping was used to examine the OH generation from the Cu(II)/5-ASA reaction. It has been shown that the interaction of hydroxyl radical with DMSO results in the formation of a CH_3 (Rosen and Rauckman, 1984 and Stoyanovsky et al., 1999). Because of a high reaction rate constant for the spin trap α -(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) with CH_3 , POBN in conjunction with DMSO has been employed in EPR spin trapping studies to assess the formation of hydroxyl radical (Rosen and Rauckman, 1984 and Stoyanovsky et al., 1999). We therefore applied this approach to examine the formation of hydroxyl radical from the Cu(II)/5-ASA reaction. As can be seen from Fig. 5A (*line b*), the reaction of Cu(II)/POBN led to formation of a POBN- CH_3 spin adduct with spectra characteristic of methyl radicals as a manifestation of OH. POBN itself did not give rise to any detectable spin adducts (Fig. 5A, (*line a*)), indicating the high purity of the spin trap used in this study. It was found that the POBN- CH_3 adduct could be detected from the reaction of Cu(II)/5-ASA at $\sim 34\%$ significantly increased intensity (Fig. 5A and B, (*line c*)). When BCS and catalase were added, the POBN- CH_3 adduct could not be detected (Fig. 5A, (*lines d and e*)); when heat-inactivated catalase was added, the spin adduct was detected at the significantly increased intensity equal to that detected in the reaction of Cu(II)/5-ASA. These observations show that both the interaction of Cu(II)/POBN and the reaction of Cu(II)/5-ASA are dependent upon Cu(II)/Cu(I) redox and H_2O_2 formation, and that the reaction of Cu(II)/5-ASA significantly increases the production of OH. Further, this finding provides definitive evidence that the reaction of Cu(II)/5-ASA potently induces OH formation responsible for the observed oxidative DNA damage.

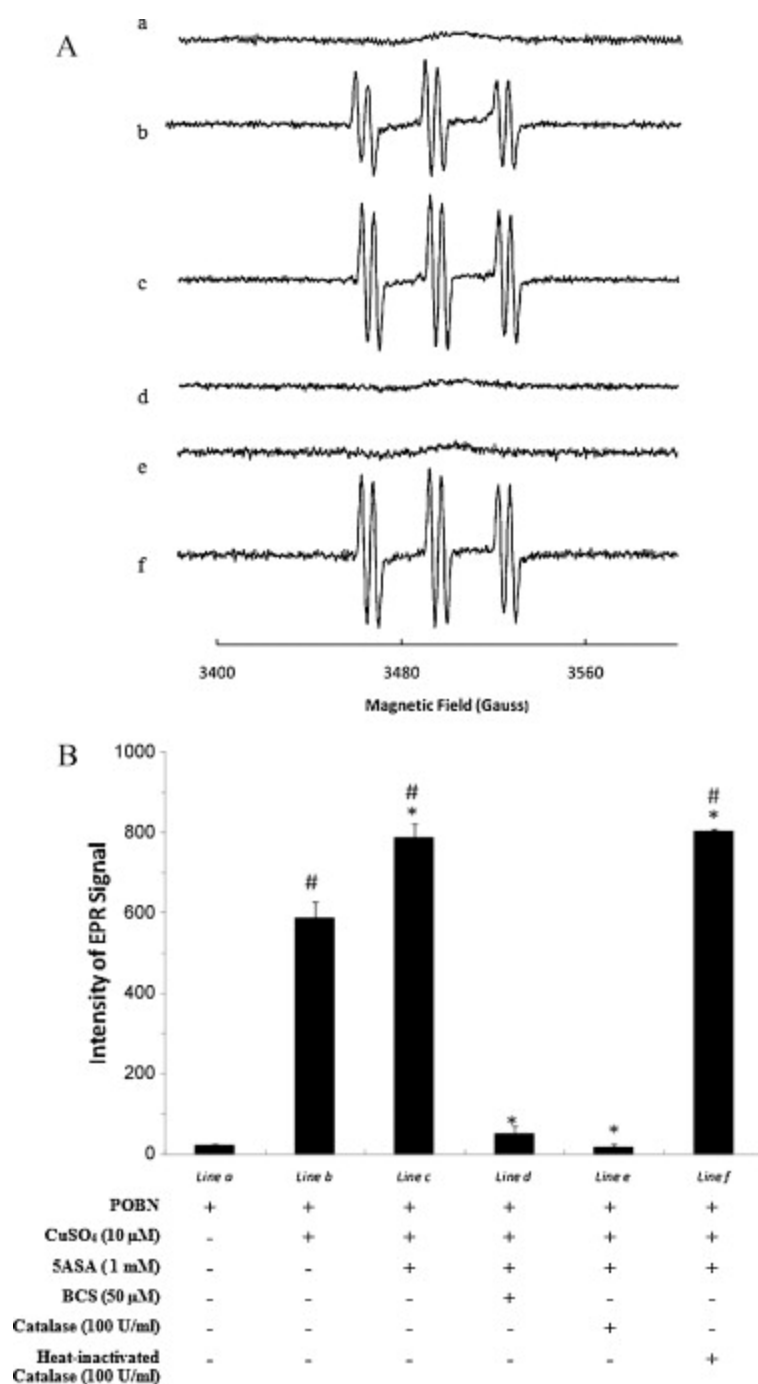


Fig. 5. Formation of hydroxyl radical from mesalamine/copper. EPR spectroscopy in combination with DMSO and the spin probe POBN were used to examine the free radicals generated by the reaction of mesalamine and copper in the presence or absence of BCS, catalase, and heat-inactivated catalase. (A) Representative EPR spectra of POBN-CH₃ adduct observed during the reaction of the reaction of 50 mM POBN, 0.5 M DMSO, and the indicated concentrations of the following experimental reagents: *Line a*, reaction buffer; *line b*, copper; *line c*, copper, 5-ASA; *line d*, copper, 5-ASA, BCS; *line e*, copper, 5-ASA, catalase; *line*

f, copper, 5-ASA, heat-inactivated catalase. EPR measurement conditions are described in Section 2. (B) Signal intensity at 3485 G expressed as mean \pm SD of three independent experiments. ([#]*P* < 0.05 vs. *line a*, **P* < 0.05 vs. *line b*.)

Cumulative evidence over the last two decades has demonstrated that reactive oxygen species, especially hydroxyl radical produced by both endogenous and exogenous sources are able to cause oxidative DNA damage in cancer cells (Hadi et al., 2007 and Lau et al., 2008). Therefore, the pro-oxidant action of 5-ASA in the presence of Cu(II) observed in this study (Fig. 2, Fig. 3, Fig. 4 and Fig. 5) may be an important mechanism for its anticancer property. Since copper is naturally associated with DNA in the nuclei (Hadi et al., 2007 and Li et al., 1994), it is reasonable to propose that the 5-ASA/copper system may also cause similar oxidative DNA strand breakage in cancer cells. Studies are currently underway to investigate the copper-dependent DNA cleaving effects of 5-ASA using cancer cell lines as an in vitro model.

4. Summary and conclusion

In summary, we have shown for the first time that the reaction of copper/mesalamine potentiates oxidative DNA damage through a mechanism involving copper redox, consumption of oxygen, production on H₂O₂, and culminating in the production potent hydroxyl radicals. Substantial evidence suggests that copper is sequestered in neoplastic cells and this study demonstrates that at pharmacologically relevant (Frieri et al., 1999) concentrations of mesalamine react with copper to potentiate hydroxyl radical formation resulting in oxidative DNA damage. This finding demonstrates a potential mechanism of the anticancer effects of mesalamine in patients with ulcerative colitis and has implications in the creation of novel anticarcinogenic compounds.

Conflicts of interest

There are none.

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